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Inhibition of squalene epoxidase by allylamine antimycotic compounds

A comparative study of the fungal and mammalian enzymes

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The inhibition of squalene epoxidase by the allylamine antimycotic agents naftifine and compound SF 86-327 was investigated, with particulate enzyme preparations from the pathogenic yeasts *Candida albicans* and *Candida parapsilosis* and from rat liver. Both naftifine and compound SF 86-327 were potent inhibitors of the *Candida* epoxidases and showed apparently non-competitive kinetics with respect to the substrate squalene. The K_i values for naftifine and compound SF 86-327 in the *C. albicans* system were 1.1 μM and 0.03 μM respectively. The *C. parapsilosis* enzyme was slightly more sensitive to inhibition. Varying the concentrations of cofactors or the soluble cytoplasmic fraction (S_{200}) had no effect on the inhibition. The epoxidase from rat liver was much less sensitive (K_i for compound SF 86-327 was 77 μM). The inhibition was also qualitatively different from that in *Candida*, being competitive with respect to squalene and also with respect to the S_{200} fraction. S_{200} fraction derived from *C. albicans* also antagonized the inhibition of the epoxidase from liver, but the liver S_{200} fraction did not affect inhibition of the *Candida* enzyme by compound SF 86-327. There was no evidence for an irreversible or mechanism-based inhibition of either the fungal or the mammalian epoxidase. The selective inhibition of squalene epoxidase was sufficient to account for the known antimycotic action of the compounds.

The allylamines are a newly developed class of antifungal agents with activity against a wide range of fungi pathogenic to man. They include the topical antimycotic naftifine (Georgopoulos *et al.*, 1981) and the more potent, systemically active, compound SF 86-327 (Petranyi *et al.*, 1984; Stuetz & Petranyi, 1984).

Naftifine was found to block ergosterol biosynthesis in several fungi, acting at the point of squalene epoxidation (Paltauf *et al.*, 1982; Ryder & Troke, 1982; Ryder *et al.*, 1984). All the available evidence indicates that inhibition of ergosterol biosynthesis is the primary mode of action of both naftifine and compound SF 86-327 (Ryder, 1984, 1985*a*). Squalene epoxidase is an essential step in the biosynthesis both of ergosterol in fungi and of cholesterol in mammals. However, experiments with rat liver cell-free extracts (Ryder, 1985*a*) indicated that cholesterol biosynthesis was several orders of magnitude less sensitive to the allylamines than was the fungal system.

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Squalene epoxidase (EC 1.14.99.7) is a microsomal enzyme catalysing the conversion of squalene into 2,3-oxidosqualene, which is then cyclized by a subsequent enzyme to form lanosterol. The epoxidase from rat liver has been the subject of extensive investigations (Yamamoto & Bloch, 1970; Tai & Bloch, 1972; Ono & Bloch, 1975; Ferguson & Bloch, 1977; Friedlander *et al.*, 1980). After solubilization, the epoxidase was shown to consist of an NADPH–cytochrome *c* reductase activity (Ono *et al.*, 1977) and a terminal oxidase, which has been purified by Ono *et al.* (1982). Much less is known about squalene epoxidase in fungi. Particulate enzyme preparations have been described from *Saccharomyces cerevisiae* (Jahnke & Klein, 1983) and the pathogenic yeast *Candida albicans* (Ryder & Dupont, 1984). The aim of the present study was to determine the enzymic basis for the antifungal activity of the allylamines and for their high degree of selectivity. To this end, we describe here the properties of the squalene epoxidase inhibition in two pathogenic *Candida* strains, of differing susceptibility to the allylamines (Georgo-

poulos *et al.*, 1981; Ryder, 1985*a*), in comparison with similar microsomal enzyme preparations from rat liver.

Parts of this investigation have been summarized in a recent review of allylamine action (Ryder, 1984).

Materials and methods

Materials

Biochemicals were purchased from Sigma Chemical Co. [4,8,12,13,17,21-^{3}H]Squalene (sp. radioactivity 3.9Ci/mmol) was obtained from New England Nuclear and purified as described previously (Ryder & Dupont, 1984). [4-^{14}C]-Cholesterol (sp. radioactivity 58.4Ci/mol) was from Amersham International. Naftifine [(*E*)-*N*-methyl-*N*-(3-phenylprop-2-enyl)-1-naphthalenemethanamine hydrochloride] and compound SF 86-327 [(*E*)-*N*-(6,6-dimethylhept-2-en-4-ynyl)-*N*-methyl-1-naphthalenemethaneamine hydrochloride] were synthesized by A. Stütz (Sandoz Forschungsinstitüt, Vienna, Austria). Rats were purchased from Zentralinstitut für Versuchstierzucht (Hanover, Germany).

Preparation of fungal squalene epoxidase

A washed microsomal fraction (200000*g* pellet) and the soluble cytoplasm (S_{200} fraction) were prepared from cells of *Candida albicans* strain Δ63 as described by Ryder & Dupont (1984). Similar fractions were prepared in the same way from *Candida parapsilosis* strain A.T.C.C. 46589. The microsomal fractions were employed as the source of enzyme. All preparations were made in 0.1M-potassium phosphate buffer, pH7.4, containing 0.5mM-dithiothreitol (subsequently referred to as 'buffer').

Preparation of liver squalene epoxidase

Livers were removed from freshly killed male Lewis rats (12–16 weeks old). Cell-free extracts (10000*g* supernatant) were prepared in buffer by the method of Ryder (1985*a*). The microsomal fraction and S_{200} fraction were then isolated as from *Candida*.

Squalene epoxidase assay

Enzyme preparations were stored in plastic ampoules in liquid N_2 and were diluted to the required protein concentration (usually 4mg/ml) immediately before use. The assay of enzyme from *Candida* was performed as described previously (Ryder & Dupont, 1984). In brief, microsomal fraction was incubated with S_{200} fraction or buffer, 1mM-NADH, 0.1mM-FAD and 4μM-[^{3}H]squalene for 1h at 30°C. Inhibitors were added in dimethyl sulphoxide (final concn. 1%, v/v) and were preincubated for 10min with the assay mixture before addition of the substrate. After saponification, the radiolabelled products (squalene 2,3-epoxide and sterols) were separated by t.l.c. and counted for radioactivity. [^{14}C]Cholesterol was used as an internal standard. All experiments employed three replicate incubations per treatment. The assay with liver enzyme was identical, except that NADPH was the cofactor. Epoxidase activity is expressed in units of nmol of squalene converted/h. Enzyme kinetic values and plots were generated by using a curve-fitting program in a Hewlett–Packard HP41C calculator.

Protein determination

The sample (0.2ml) was mixed with 0.2ml of 1M-NaOH and left 10min at room temperature. The mixture was then diluted with 1.6ml of distilled water. Samples (0.1ml) were removed and assayed with the Bio-Rad reagent according to the supplier's instructions, with bovine γ-globulin as standard.

Results

Comparative properties of squalene epoxidase preparations

Table 1 shows a comparison of the fungal and rat liver enzymes. The enzyme obtained from *C. parapsilosis* was similar in properties to that from *C. albicans* previously described by Ryder & Dupont (1984). Activity was associated with particulate cell fractions, required NADH and FAD, and was stimulated by the S_{200} fraction, which itself contained negligible epoxidase activity. The assay was linear with time up to 90min, with or without S_{200} fraction. In the presence of S_{200} fraction, activity was linear with microsomal protein concentration up to 1.2mg/ml of assay mixture. In the absence of S_{200} fraction, activity was disproportionately low at low enzyme concentrations, as previously shown in *C. albicans* (Ryder & Dupont, 1984).

The microsomal squalene epoxidase from rat liver was previously characterized by Yamamoto & Bloch (1970) and Tai & Bloch (1972). The assay system used in the present study was linear with time to 90min and with microsomal protein concentration to 1.6mg/ml. The apparent K_m for squalene of 11μM (Table 1) was similar to that published for the purified rat liver epoxidase (Ono *et al.*, 1982). In contrast with the *Candida* enzyme, the epoxidase from rat liver was virtually inactive in the absence of S_{200} fraction (Table 1), in agreement with previous work (Yamamoto & Bloch, 1970). The effect of S_{200} fraction was concentration-dependent, and Lineweaver–Burk plots gave an an apparent K_m of 24±14mg/ml (mean±S.D. for seven separate experiments). The apparent K_m for S_{200} fraction was not affected by

Table 1. *Comparative properties of squalene epoxidase preparations from Candida and from rat liver*
Results refer to the standard enzyme assay, except for kinetic data, which were obtained as described by Ryder & Dupont (1984). Results for *C. albicans* are from Ryder & Dupont (1984).

Property	*C. albicans* enzyme	*C. parapsilosis* enzyme	Rat liver enzyme
K_m for squalene (μM)	50	16	11
$V_{max.}$ (units/mg of protein)	5.8	2.4	2.4
Preferred cofactor	NADH	NADH	NADPH
Absence of S_{200} (% of activity with S_{200})	$\geqslant 50\%$	$\geqslant 50\%$	$<10\%$
0.1 mM-2-Heptyl-4-hydroxyquinoline *N*-oxide (% of control)	236.4	96.2	78.1
0.1 mM-Rotenone (% of control)	32.7	45.7	79.1

Table 2. *Inhibition of fungal and mammalian squalene epoxidase by compound SF 86-327 and naftifine*
The concentrations required to inhibit squalene epoxidase activity under standard assay conditions by 50% (I_{50}) or by 95% (I_{95}) were determined from plots of percentage of enzyme activity remaining versus the logarithm of the concentration of inhibitor (Fig. 1). Results are expressed as means ± S.D. (numbers of independent experiments in parentheses). K_i values were determined from kinetic plots (as shown in Figs. 2 and 3) as described by Segel (1975), and are derived from at least three experiments in each case.

	Compound SF 86-327			Naftifine	
Source of enzyme	K_i (μM)	I_{50} (μM)	I_{95} (μM)	I_{50} (μM)	I_{95} (μM)
C. albicans	0.03	0.030 ± 0.008	2.10 ± 0.85 (5)	1.1 ± 0.2	30.9 ± 3.1 (5)
C. parapsilosis	0.04	0.040 ± 0.012	0.55 ± 0.06 (6)	0.34 ± 0.15	6.1 ± 3.2 (5)
Rat liver	77	93 ± 30	> 300 (3)	144 ± 18	> 300 (3)

squalene concentration. The S_{200} fraction derived from *C. albicans* cells could fully substitute for the rat liver S_{200} fraction.

Inhibition of fungal squalene epoxidase by allylamines

The effects of naftifine and compound SF 86-327 were examined in detail in the *C. albicans* epoxidase system. Both compounds caused a concentration-dependent inhibition, which became total at low concentrations of either compound, compound SF 86-327 being the more potent inhibitor (Fig. 1). The degree and range of inhibition by naftifine and compound SF 86-327 were completely unaffected by the presence or absence of S_{200} fraction (Fig. 1). Rat liver S_{200} fraction also stimulated the *C. albicans* epoxidase, but had no effect on the degree of inhibition by the allylamines. Table 2 summarizes the quantitative results obtained. Dixon plots at different substrate (squalene) concentrations were typical of non-competitive-type inhibition (Segel, 1975). This is shown in Fig. 2 for naftifine, with K_i 1.1 μM; a similar result was obtained with compound SF 86-327, giving K_i 0.03 μM (Petranyi *et al.*, 1984). Kinetic results were obtained in the presence of S_{200} fraction, which enables the use of higher

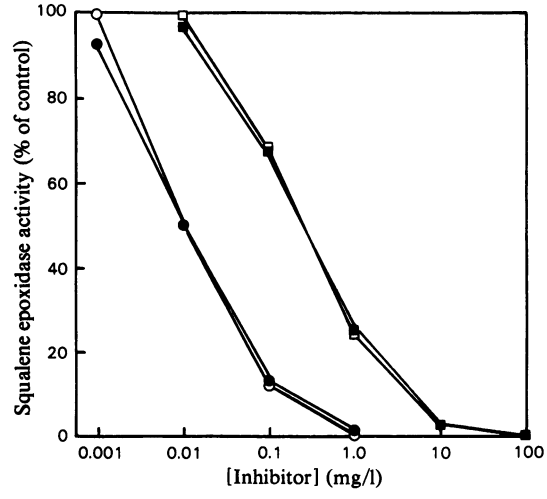

Fig. 1. *Effects of naftifine and compound SF 86-327 on squalene epoxidase from C. albicans microsomal fraction*
Squalene epoxidase activity was determined by using the standard assay as described in the Materials and methods section, with increasing concentrations of compound SF 86-327 in the presence (○) or in the absence (●) of S_{200} fraction, or with increasing concentrations of naftifine in the presence (□) or in the absence (■) of S_{200} fraction.

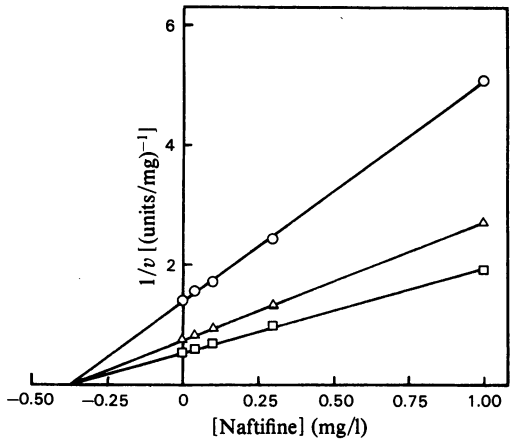

Fig. 2. *Dixon analysis of naftifine inhibition of C. albicans squalene epoxidase in the presence of increasing concentrations of squalene*

Squalene epoxidase activity was determined in the presence of various naftifine concentrations, with as substrate 14.7 μM- (○), 32.2 μM- (△) or 116.9 μM-squalene (□).

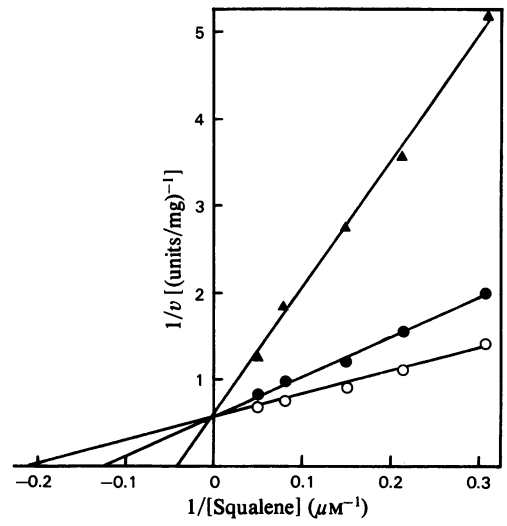

Fig. 3. *Lineweaver–Burk analysis of inhibition by compound SF 86-327 of rat liver squalene epoxidase over a range of squalene concentrations*

Squalene epoxidase activity was determined over a range of squalene concentrations, in the absence (○) or in the presence of compound SF 86-327 at 30 mg/l (●) or 100 mg/l (▲).

squalene concentrations in the assay (Ryder & Dupont, 1984). Increasing the concentrations of FAD and NADH up to 10-fold, or substituting NADPH for NADH, had no influence on the inhibition. Enzyme preparations treated with 2-heptyl-4-hydroxyquinoline *N*-oxide to stimulate epoxidase activity (Ryder & Dupont, 1984) were equally sensitive to the action of compound SF 86-327.

No time-dependent inhibition was observed when the enzyme was preincubated for periods between 0.5 and 60 min in the presence of compound SF 86-327 and cofactors before dilution into the assays. Similarly, addition of compound SF 86-327 to epoxidase assays resulted in an apparently instantaneous inhibition, which remained constant during subsequent incubation. The degree of inhibition always corresponded to the end concentration of compound and was unaffected by preincubation with higher concentrations.

In comparison with the *C. albicans* enzyme, the squalene epoxidase from *C. parapsilosis* was slightly more sensitive to inhibition by the allylamines, more noticeably in the case of naftifine (Table 2). In all other respects, the inhibition was similar to that in *C. albicans*, being non-competitive with respect to squalene and unaffected by S_{200} fraction or cofactor concentration, or by preincubation with the inhibitor.

Effect of allylamines on rat liver squalene epoxidase

The rat liver enzyme was much less sensitive to the allylamines than the fungal epoxidases, by a factor of about three orders of magnitude (Table 2). Full inhibition of activity could not be achieved within the solubility limits of the two compounds. The difference in potency between naftifine and compound SF 86-327 was also much less marked than in the fungal systems (Table 2). Only the more potent compound SF 86-327 was investigated in detail. Kinetic analysis (Fig. 3) showed a competitive inhibition with respect to squalene concentration. In further contrast with the *Candida* epoxidase, the concentration of S_{200} fraction influenced the effect of compound SF 86-327, in an apparently competitive manner (Fig. 4). The *C. albicans* S_{200} fraction also antagonized inhibition of the rat liver enzyme. Inhibition was not antagonized by increased concentrations of NADPH and FAD or by supplying NADH as cofactor.

Within the limits of detection of the assay system, inhibition occurred immediately upon adding compound SF 86-327 and remained constant during a further 60 min incubation. The reversible nature of the inhibition was confirmed by experiments involving preincubation of enzyme with compound SF 86-327 and cofactors, followed by dilution and epoxidase assay. Table 3 shows results of a typical experiment, in which the degree of inhibition corresponded to the final concentration of compound SF 86-327 in the assay, and was unaffected by up to 90 min preincubation with a 5-fold higher concentration of the drug.

Table 3. *Reversibility of inhibition of rat liver squalene epoxidase by compound SF 86-327*
Rat liver microsomal fraction (protein concn. 2mg/ml) was preincubated with cofactors and S_{200} fraction (as for standard assay) and with compound SF 86-327 (50mg/l). After the time shown, 100 µl samples were removed and assayed for squalene epoxidase activity by the standard method with 0.5ml assay volume. For comparison, standard assays with 10min preincubation were performed with the relevant concentrations of microsomal protein and compound SF 86-327. In all cases, inhibition by compound SF 86-327 is expressed as a percentage of the enzyme activity of controls run in parallel. Results are means for triplicate incubations.

Treatment	Preincubation (min)	Final concn. of microsomal protein (mg/ml)	Final concn. of compound SF 86-327 (mg/ml)	Inhibited activity (% of control)
Standard assay	10	2.0	50	47.0
Standard assay	10	0.4	50	54.3
Diluted 1:5	10	0.4	10	18.2
Diluted 1:5	90	0.4	10	22.3
Standard assay	10	0.4	10	22.6

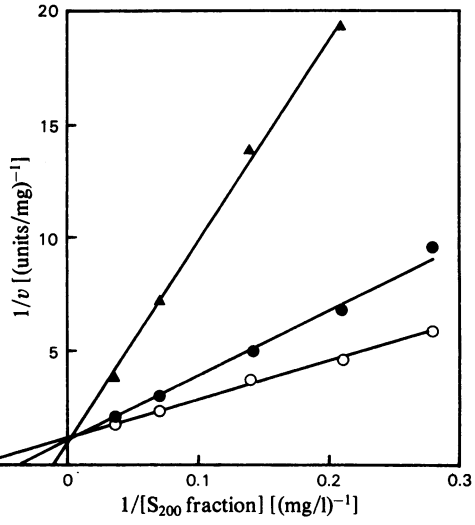

Fig. 4. *Lineweaver–Burk analysis of inhibition by compound SF 86-327 of rat liver squalene epoxidase over a range of concentrations of soluble cytoplasm*
Squalene epoxidase activity was determined over a range of concentrations of S_{200} fraction, in the absence (○) or in the presence of compound SF 86-327 at 30mg/l (●) or 100mg/l (▲).

Discussion

Our study clearly demonstrates that both naftifine and SF 86-327 are potent inhibitors of squalene epoxidase in *Candida*. This action is sufficient to account for the previously described inhibition of ergosterol biosynthesis in *Candida* cells caused by naftifine (Ryder, 1984; Ryder *et al.*, 1984) and compound SF 86-327 (Ryder, 1985*a*). No evidence could be found for significant inhibition by compound SF 86-327 of other enzymes in the ergosterol pathway (Ryder, 1985*a*). Naftifine had secondary effects on sterol-side-chain methylation only at concentrations much higher than those required for inhibition of squalene epoxidase (Ryder, 1985*b*). The specific inhibition of squalene epoxidase thus seems to constitute the primary mechanism of the antifungal action of the allylamines. As far as we are aware, no other specific inhibitors of this enzyme have been reported.

The relative activities of naftifine and compound SF 86-327 as epoxidase inhibitors correlate with the antifungal efficacy of the two compounds (Ryder, 1984). However, the observed differences in sensitivity of the epoxidase in the two *Candida* strains do not appear sufficient to explain the fact that *C. parapsilosis* is more susceptible than *C. albicans* to growth inhibition by either compound, by a factor of one to two orders of magnitude. Dermatophyte fungi are even more susceptible to the allylamines (Petranyi *et al.*, 1984). Susceptibility would appear to be determined, not only by sensitivity of the epoxidase, but also by physiological properties of the fungus (Ryder, 1985*a*) and the extent of available sterol precursors in the fungal cell (Ryder, 1985*b*).

The present study shows that the high degree of selectivity of action of the allylamines is due to differential sensitivity of the fungal and mammalian squalene epoxidases. In the case of compound SF 86-327, this difference operates by a factor of over three orders of magnitude, greatly enhancing the chemotherapeutic usefulness of the compound as a systemically acting drug. Inhibition of the mammalian epoxidase appears to be qualitatively different from that in *Candida*, being competitive with the substrate squalene and antagonized by the soluble cytoplasm (S_{200} fraction). A further difference lies in the structural specificity required for enzyme inhibition; in *Candida*, compound SF 86-327 was about 30-fold more active than naftifine as an inhibitor, whereas there was little difference between the two compounds in the rat liver system. Various structurally related compounds devoid of antifungal activity show a quantitatively similar

degree of inhibition of the rat liver epoxidase (N. S. Ryder & M.-C. Dupont, unpublished work), underlining the non-specific nature of the effect of the allylamines in this system.

Care must be exercised in interpreting the results of kinetic experiments with membrane-bound enzymes. However, the results are consistent with the allylamines having a relatively low affinity for the squalene-binding site on the mammalian epoxidase, thus resulting in a reversible competitive inhibition. A variety of isoprenoid compounds can inhibit the rat liver epoxidase, presumably by competing with squalene (Morin & Srikantaiah, 1982). Alternatively, the inhibitors could compete for the stimulatory soluble cytoplasmic factor. The role of this factor is not entirely clear, being variously described as a 'carrier protein' (Srikantaiah *et al.*, 1976) or an aid to the inter-membrane transport of squalene (Friedlander *et al.*, 1980). The *C. albicans* S_{200} fraction could substitute for the rat liver S_{200} fraction, in agreement with an earlier report (Dempsey & Meyer, 1977) of the isolation from yeast cells of a soluble protein that stimulated rat liver cholesterol biosynthesis.

The highly specific non-competitive inhibition of the *Candida* epoxidase is unlikely to be due to the allylamines acting as substrate analogues. Furthermore, the inhibition is reversible in both the fungal and mammalian enzymes, as was also demonstrated in whole fungal cells (Ryder *et al.*, 1984). These features would rule out a mechanism-based inhibition involving the acetylenic function of compound SF 86-327, such as described for the propargyline derivatives that inhibit monoamine oxidase (Maycock *et al.*, 1976). The high selectivity suggests that allylamines may inhibit a component of the epoxidase system that is specific to fungi. The most obvious difference between the fungal and mammalian enzymes is in the effect of S_{200} fraction, but this appeared to play no role in the inhibition of the fungal epoxidase. Further potential sites of action would be the electron-transport supply to the epoxidase, which differs with respect to cofactors and sensitivity to inhibitors, and the lipid environment of the membrane-bound epoxidase. Purification of the components of the fungal squalene epoxidase will be necessary to resolve this question.

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